# EXHIBIT I

housekeeping protein. The absence of typical leader peptide from the deduced amino acid sequence (*11*) may suggest that the protein is not destined to penetrate the endoplasmic reticulum and therefore its potential *N*-linked glycosylation site (Fig. 1) may not be used. The strong interspecies conservation of the WL623 nucleotide sequence, and the even more rigidly preserved dipeptide repeat element as well as the widespread expression of the gene, provide strong evidence that the protein subserves a fundamental and probably phylogenetically ancient function. The most striking feature of this previously undetected gene resides in its central repeat element for which we have failed to find a significant match in the protein sequences available from different databases. Moreover, our inability to relate any segment of the remaining sequence to that of other proteins (*12*) indicates that WL623 identifies a novel class of proteins. Finally our finding adds a new element in support of the genetic heterogeneity of the class III region of the MHC, a concept that already emerged with the cloning of the cytochrome P450 gene encoding the steroid 21 hydroxylase isozyme (*13*).


REFERENCES AND NOTES

1. J. Klein, Ed., *Natural History of the Major Histocompatibility Complex* (Wiley, New York, 1986).
2. U. Muller, D. Stephan, P. Philippsen, M. Steinmetz, *EMBO. J.* 6, 369 (1985); M. Tosi, M. Lévi-Strauss, E. Georgatsou, M. Amor, T. Meo, *Immunol. Rev.* 87, 151 (1985).
3. M. Lévi-Strauss, M. Carroll, M. Steinmetz, T. Meo, in *Major Histocompatibility Genes and Their Role in Immune Function* (Plenum, New York, in press).
4. M. Steinmetz *et al.*, *EMBO. J.* 3, 2995 (1984).
5. S. M. Mount, *Nucleic Acids Res.* 10, 459 (1982).
6. M. Kozak, *ibid.* 12, 857 (1984).
7. S. Ohno and J. T. Epplen, *Proc. Natl. Acad. Sci. U.S.A.* 80, 3391 (1983).
8. M. Lévi-Strauss, E. Georgatsou, M. Tosi, T. Meo, *Immunogenetics* 21, 397 (1985).
9. M. Lévi-Strauss, M. Tosi, M. Steinmetz, J. Klein, T. Meo, *Proc. Natl. Acad. Sci. U.S.A.* 82, 1746 (1985).
10. M. Carroll, D. R. Bentley, R. R. Porter, *Nature (London)* 307, 237 (1984).
11. M. E. E. Watson, *Nucleic Acids Res.* 12, 5145 (1984).
12. An exhaustive homology search was run using an adaptation of the Lipman-Pearson FASTP program [D. J. Lipman and W. R. Pearson, *Science* 227, 1435 (1985)], as well as the "explor" program [J. M. Claverie and L. Sauvaget, *Comput. Appl. Biosci.* 1, 85 (1985)] on the PSEQIP data bank [J. M. Claverie and L. Bricault, *Proteins* 1, 60 (1986)].
13. M. Amor, M. Tosi, C. Duponchel, M. Steinmetz, T. Meo, *Proc. Natl. Acad. Sci. U.S.A.* 82, 4453 (1985); P. C. White *et al.*, *Nature (London)* 312, 465 (1984).
14. Sequence determination was performed as described by F. Sanger, S. Nicklen, and A. R. Coulson [*Proc. Natl. Acad. Sci. U.S.A.* 74, 5463 (1977)], by using the single-strand deletion method of R. M. K. Dale, B. A. McClure, and J. P. Houchins [*Plasmid* 13, 31 (1985)]. Sequence ambiguities were removed by using the following three synthetic oligonucleotides (symbolized by a dot in Fig. 1C): positions 385 → 401; 726 → 742; and 892 ← 876.
15. M. L. Birnstiel, M. Busslinger, K. Strub, *Cell* 41, 349 (1985). Preliminary data rule out that this anomaly results from a cloning or sequencing artifact.
16. H. Land, M. Grez, H. Hauser, W. Lindenmaier, G. Schütz, *Nucleic Acids Res.* 9, 2251 (1982).
17. *Immunol. Rev.* 87 (1985), entire issue.
18. T. Maniatis, E. F. Fritsh, J. Sambrook, in *Molecular Cloning* (Cold Spring Harbor Laboratory, Cold Spring Harbor, NY, 1982).
19. A. P. Feinberg and B. Vogelstein, *Anal. Biochem.* 132, 6 (1983).
20. S. Szpirer and J. Szpirer, *J. Differentiation* 4, 85 (1975).


21. We thank J. M. Claverie for discussions and computing help and A. Bernardin and C. Verna for their kind assistance in the preparation of the manuscript.

15 October 1987; accepted 29 February 1988


# Genetically Transformed Maize Plants from Protoplasts


CAROL A. RHODES, DOROTHY A. PIERCE,* IRVIN J. METTLER, DESMOND MASCARENHAS,† JILL J. DETMER†



**Genetically transformed maize plants were obtained from protoplasts treated with recombinant DNA. Protoplasts that were digested from embryogenic cell suspension cultures of maize inbred A188 were combined with plasmid DNA containing a gene coding for [illegible] (NPT II) next to the 35S promoter region of cauliflower mosaic virus. A high voltage electrical pulse was applied to the protoplasts, which were then grown on filters placed over feeder layers of maize suspension cells (Black Mexican Sweet) and selected for growth in the presence of kanamycin. Selected cell lines showed NPT II activity. Plants were regenerated from transformed cell lines and grown to maturity. Southern analysis of DNA extracted from callus and plants indicated the presence of the NPT II gene.**


AGRICULTURALLY IMPORTANT CEREAL crops, including maize, have been difficult to engineer genetically by current techniques for gene insertion. With few exceptions (*1*, *2*), most of the graminaceous crops are not readily susceptible to infection by *Agrobacterium tumefaciens*, which is a vector for gene transfer commonly used with many dicot species (*3*, *4*). Genes can be transferred directly into protoplasts, without an *Agrobacterium* vector, by methods that permit DNA to cross the plasmalemma (*5*–*8*). Stable transformation of maize cells has been achieved through direct uptake of DNA into protoplasts that had been permeabilized by electroporation (*8*, *9*), but until recently (*10*) no plants had been recovered from maize protoplasts. We now describe regeneration of maize plants derived from protoplasts into which a gene encoding neomycin phosphotransferase II (NPT II) was introduced via electroporation. NPT II permits plant cells to grow on inhibitory levels of the antibiotic kanamycin (*3*, *8*) and can be used as a dominant marker to select for transformed cells.

Protoplasts were isolated from an embryogenic cell suspension culture of maize inbred A188 (*10*). The cell culture was initiated as callus from immature embryos 18 months before these experiments were begun and had been grown as a suspension culture in liquid N6ap medium (*10*) for approximately 1 year. Plants were then easily regenerated by transferring callus to N6ap or MS (*11*) medium without auxin.

Freshly prepared protoplasts were suspended at densities of $3 \times 10^6$ to $6 \times 10^6$ per milliliter of N6ap medium (450 mosM,

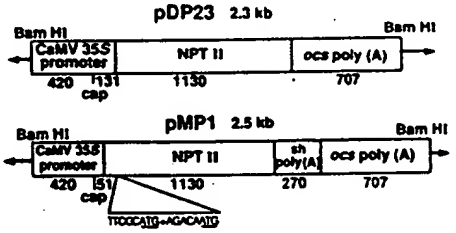


**Fig. 1.** Diagram of pDP23 and pMP1. The 35*S* promoter fragment in pDP23 (*9*) extends from the Bam HI site at −420 bp to the Dde I site at +131 bp with respect to the start of RNA transcription (*12*). The fragment carrying the NPT II gene includes 15 bp of 5′ noncoding region and 343 bp of 3′ noncoding region extending to the Sal I site in Tn5. The false start ATG 16 bp upstream from the original Tn5 version has been deleted. The 3′ region of the *ocs* gene was recovered as a 707-bp Pvu II fragment (*13*). The cassette in pMP1 contains a version of the 35*S* promoter that includes 51 bp of CaMV sequence downstream from the start of RNA transcription (*9*). For pMP1, the sequence in the 5′ noncoding region of NPT II in pDP23 was changed from 5′-TTCGCATG-3′ to 5′-AGACA ATG-3′. The 3′ region of pMP1 includes a 270-bp Rsa I fragment from the 3′ region of the sucrose synthase (sh) gene (*19*) fused at its 3′ end to the Pvu II fragment of the *ocs* polyadenylation region. The NPT II cassettes in pDP23 and pMP1 are bordered by Bam HI sites and were inserted into the Bam HI site of pUC19.


Sandoz Crop Protection Corporation, Palo Alto, CA 94304-1104.

*Present address: EniChem, Monmouth Junction, NJ 08852.

†Present address: BioGrowth, Richmond, CA 94806.

*p*H 8.0). In some experiments, protoplasts were incubated for 2 to 4 minutes at 43°C and then cooled by ice water before proceeding. This heat treatment did not affect protoplast viability. Protoplasts were portioned into microfuge tubes (0.5 ml per tube) and the following solutions were added to each tube: 75 μl of 2*M* KCl; 20 μl of sonicated calf thymus DNA (2.5 mg/ml); 0.5 ml of N6ap medium, *p*H 8.0; and 20 to 40 μg of supercoiled plasmid DNA.

Two plasmid constructions, pDP23 and pMP1 (Fig. 1), were used, each of which included a bacterial NPT II gene from Tn5 as a selectable marker for transformation. Plasmid pDP23 (*9*) contained the 35*S* promoter region of cauliflower mosaic virus (CaMV) (*12*), including 131 base pairs of untranslated leader sequence and the 3′ polyadenylation region of the octopine synthase gene (*ocs*) from the Ti plasmid of *Agrobacterium tumefaciens* (*13*). The pMP1 plasmid contained a similar version of the CaMV 35*S* promoter, but with only 51 base pairs of leader sequence (*9*) and selected changes in the sequence upstream from the ATG start signal in the NPT II coding region, as well as a modified 3′ terminal region (Fig. 1).

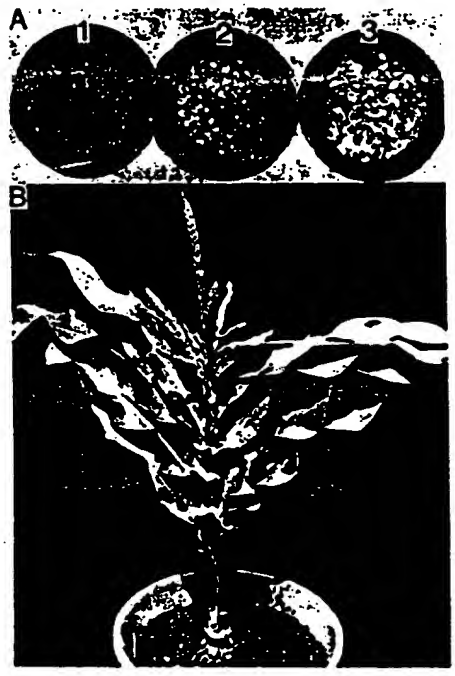


**Fig. 2.** Selection of kanamycin-resistant A188 callus and regeneration of plants. A188 protoplasts were electroporated in a solution containing the plasmid pMP1 (20 to 40 μg/ml) and selected for growth in the presence of kanamycin, as described in the text. (**A**) Callus grown from electroporated protoplasts after 19 days on medium containing kanamycin. Filter 1 shows minimal growth from control protoplasts, which were electroporated without pMP1. Filter 2 shows callus grown from protoplasts that were electroporated in the presence of pMP1. Protoplasts on filter 3 were subjected to a heat treatment (2 minutes at 43°C) before electroporation under the same conditions used with protoplasts on filter 2. (**B**) Mature plant regenerated from protoplast-derived callus selected for kanamycin resistance. As is typical for regenerated maize plants, this plant was about half as tall as seed-grown plants.

Each mixture of protoplasts and DNA was immediately transferred to sterile 0.4-cm plastic cuvettes fitted with aluminum foil electrodes (*9*, *14*). An electrical field of 200 to 300 V (500 to 750 V/cm) was applied at room temperature by discharge of a 1200-μF capacitor that had been previously charged with an electrophoresis power supply. Five minutes later, electroporated protoplasts were diluted with two volumes of N6ap medium (*p*H 5.8). Percentages of viable protoplasts decreased with increasing field strength. Application of 250 V (625 V/cm) caused a 30 to 50% decrease in viability.

Protoplasts were plated on Millipore filters (pore size, 0.8 μm) over N6ap medium (adjusted to 450 mos*M* with mannitol) with Black Mexican Sweet (BMS) suspension cells as a nurse culture. Each feeder layer plate consisted of 0.5 g of BMS cells suspended in 2 ml of liquid N6ap medium and spread over 20 ml of agar-solidified N6ap (*10*). Millipore support filters with protoplasts were transferred after 7 days to N6ap medium of reduced osmolarity (300 mos*M*) with fresh BMS feeder layers. Kanamycin sulfate (50 to 150 mg/liter) (Sigma) was included in the growth medium after the initial 7 days to inhibit growth of cells not expressing the NPT II gene. Filters were transferred again at day 14 to N6ap medium (180 mos*M*) with kanamycin and without any BMS feeder layers (Fig. 2A). Three weeks after electroporation, individual calli that were resistant to growth inhibition by kanamycin were selected from the filter and transferred directly to N6ap medium containing kanamycin. After an additional 2 weeks of growth, there was sufficient callus tissue (approximately 800 mg) of each kanamycin-resistant cell line to assay for NPT activity, as well as to regenerate plants (Fig. 2B). This protocol of 4 weeks of selection for resistance to kanamycin resulted in virtually no nontransformed "escapes."

Experiments were done to identify the optimum concentration of kanamycin for use in this selection system. Growth of a nontransformed suspension was nearly stopped after 3 weeks in kanamycin at 50 mg/liter, whereas the growth rate of one transformed cell suspension in kanamycin at 500 mg/liter was 77% of its growth rate without kanamycin. Low levels of NPT II expression were correlated with slow growth of some transformed cell lines on kanamycin at 150 mg/liter. There was some inhibition of BMS feeder cell growth on medium containing kanamycin at 100 mg/liter, but not enough in 7 days to visibly affect protoplast growth. We recommend using kanamycin at 100 mg/liter in this selection system, as this concentration effectively inhibits growth of control calli without requiring extremely high levels of NPT II expression in transformants.

This selection system provided immediate immobilization of protoplasts, thereby reducing the probability of individual transformed clones splitting into two or more cell lines or of protoplast fusions that could create chimeric cell lines. Only one presumptive chimeric clone was obtained among approximately 200 selected cell lines.

Transformation frequencies were calculated by comparing the number of kanamycin-resistant NPT II–positive calli to the number of calli grown without selection from replicate plates receiving the same treatment. Transformation frequencies were as high as 5% of callus-forming protoplasts. More typical frequencies were lower, approximately 0.5 to 1%. Heat treatment of protoplasts before electroporation was

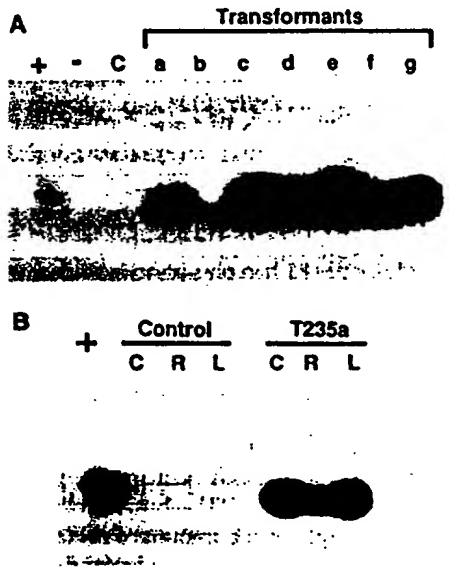


**Fig. 3.** NPT II expression in transformed A188 callus and plants. Extracts (20 μl) of callus and plant tissues were electrophoresed through a polyacrylamide gel and assayed for NPT II activity (*15*). (**A**) Extract of a BMS cell line transformed with the NPT II gene was the positive control in the first lane (+). The third lane (lane C) contained extract from A188 callus grown from protoplasts electroporated without pMP1 or pDP23 present. Lanes a through g contain extracts from kanamycin-resistant callus selected in three separate transformation experiments. (**B**) NPT II expression in transformed plants. Extracts from callus (C) and from roots (R) and leaves (L) of a transformed plant regenerated from this cell line (T235a) contained NPT II activity, whereas no NPT II activity was present in extracts of equivalent nontransformed control tissues (control lanes C, R, and L).

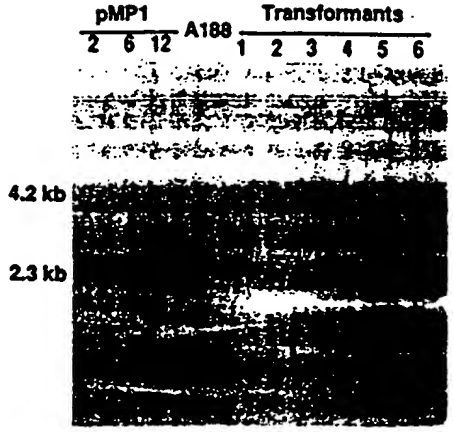


Fig. 4. Southern blot analysis of DNA from A188 callus and regenerated plants. DNA was isolated from a plant (lane 1) and callus lines (lanes 2 to 7) with NPT II activity and from control callus (A188) by modification of a previous method (*20*). Tissues were frozen in liquid nitrogen, ground to a powder, and homogenized in buffer containing 50 m*M* tris, *p*H 8.0, 50 m*M* EDTA, 250 m*M* NaCl, and 10 m*M* β-mercaptoethanol. After extraction with phenol and chloroform, polysaccharides were removed by treatment of the aqueous phase with 0.1 volume of ethanol for 10 minutes at 0°C, then centrifuging the polysaccharides at 10,000*g* for 5 minutes at 4°C (*21*). Nucleic acids were then precipitated with ethanol from the aqueous phase. The DNA was cut with the restriction enzyme Bam HI, electrophoresed through an agarose gel, and transferred to a nitrocellulose membrane (*22*). The blot was hybridized to a $^{32}$P-labeled 0.9-kb fragment that contained the NPT II gene. The first three lanes were loaded with Bam HI–restricted pMP1 at the equivalent of 2, 6, and 12 copies per haploid maize genome ($4.5 \times 10^9$ bp).

sometimes effective in increasing transformation frequency as much as threefold (Fig. 2A). This result was not consistent; less vigorous protoplasts showed no effect with heat treatment. Electroporation with 250 V (625 V/cm) produced higher transformation frequencies than electroporation with lower voltages, despite greater reductions in the percentage of viable protoplasts (30 to 50% decrease) than were seen with lower voltages.

Cell extracts of kanamycin-resistant calli and regenerated plants were tested for NPT II activity by means of a standard in vitro assay (*15*). Callus from protoplasts porated without DNA encoding NPT II showed no NPT II activity, whereas callus from protoplasts porated in the presence of either pDP23 or pMP1 had phosphorylation activity that migrated with the activity in transformed BMS callus (Fig. 3A). NPT II activity was stable in callus grown without kanamycin for at least 10 months. Relative specific activity of NPT II was calculated from liquid scintillation counting of the amount of radioisotope-labeled kanamycin phosphate bound to the P81 phosphocellulose paper, adjusted for the amount of protein in the original extract loaded per lane. The mean value for relative specific activity of NPT II from 58 cell lines transformed with pMP1 was 155, compared to a mean value of 34 from 34 cell lines transformed with pDP23. This corresponds well with relative values obtained with transient expression assays of these two plasmids in BMS protoplasts (*16*). Specific activities of NPT II in leaf and root tissues of plants were 1.8 and 2.0 times higher, respectively, than levels in transformed callus (Fig. 3B).

Southern blot analysis of DNA from callus and plants with NPT II activity was performed to confirm the presence of the NPT II gene. The 0.9-kb Pst I fragment which carries the NPT II coding sequence was isolated and labeled with $^{32}$P by nick translation, then hybridized to Bam HI–restricted genomic DNA from several maize cell lines and plant tissue (Fig. 4). The NPT II probe did not hybridize to DNA from nontransformed A188 callus, but did hybridize to the expected 2.35-kb DNA fragment of Bam HI–digested pMP1, which carries the NPT II gene cassette (lanes designated pMP1 and A188 in Fig. 4). DNA from a regenerated plant and five kanamycin-resistant cell lines, derived from three separate experiments, hybridized to the NPT II DNA probe (Fig. 4, lanes T1 through T6). The expected fragment size of 2.35 kb was not always observed. Transformants 3, 4, and 6 apparently contained the NPT II gene cassette rearranged in a way that altered the position of the Bam HI restriction sites. These three cell lines were recovered from protoplasts receiving different treatments, thus eliminating the possibility that their similar hybridization patterns were the result of sampling the same transformant more than once. None of these rearrangements correlated with relative NPT II activity in these calli. Southern blot analysis of unrestricted DNA of these transformants revealed no hybridization to the NPT II gene probe at the expected size of single-copy plasmids, an indication that the transforming DNA was integrated into the plant genomes. The estimated number of NPT II gene copies was less than five per transformant genome, as judged by a comparison with copy number standards included on the same hybridized filter (Fig. 4). Relative specific activity of NPT II, which ranged from 34 to 594 for these transformants, was not directly correlated with the number of NPT II gene copies per genome.

Regeneration of shoots occurred readily in approximately 18% of the transformed calli when transferred to medium without 2,4-dichlorophenoxyacetic acid. At this time, 29 cell lines from five separate experiments have produced plants that were transferred to soil. Thirty-eight plants derived from ten different cell lines have reached maturity. None have yet produced viable pollen; only one plant produced silks, but did not produce seed when outcrossed. Insufficient numbers of control plants have grown to maturity to permit conclusions as to whether the processes of protoplasting, transformation, or the cell line itself might be responsible for lack of fertility. Efforts continue to regenerate fertile plants from these transformed cultures.

We have demonstrated that maize protoplasts can be genetically transformed and grown to mature plants that express the inserted gene. Electroporation is a convenient method of transformation, both because of its simplicity and the reasonable rates of transformation that can be achieved. Although the transformed plants from these electroporation experiments did not produce seed, it is likely that the infertility problems are associated with the cell line itself. These cultures are now over 2 years old, and it is not unusual for maize cell cultures of this age to carry genetic changes serious enough to cause sterility (*17, 18*). Other work (*10*) indicates that this technique should be applicable to other maize cell cultures and genotypes.

As it exists now, this system permits testing of tissue-specific gene promoters and of gene expression in nonchimeric maize plants. Our data on levels of NPT II activity in callus and plants transformed with 35*S* NPT II *cat* gene constructs indicate that expression in plants can be as high as or higher than in callus.

## REFERENCES AND NOTES


1. A. C. F. Graves and S. Goldman, *Plant Mol. Biol.* 7, 43 (1986).
2. N. Grimsley, T. Hohn, J. W. Davies, B. Hohn, *Nature (London)* 325, 177 (1987).
3. R. Fraley *et al.*, *Proc. Natl. Acad. Sci. U.S.A.* 80, 4803 (1983).
4. P. Zambryski, L. Herrera-Estrella, M. De Block, M. Van Montagu, J. Schell, in *Genetic Engineering*, A. Hollaender and J. Setlow, Eds. (Plenum, New York, 1984), vol. 6, pp. 253–278.
5. F. H. Krens, L. Molendijk, G. Wullems, R. A. Schilperoort, *Nature (London)* 296, 72 (1982).
6. R. D. Shillito, M. W. Saul, J. Paszkowski, M. Muller, I. Potrykus, *BioTechnology* 3, 1099 (1985).
7. C. D. Riggs and G. W. Bates, *Proc. Natl. Acad. Sci. U.S.A.* 83, 5602 (1986).
8. M. F. Fromm, L. P. Taylor, V. Walbot, *Nature (London)* 319, 791 (1986).
9. D. A. Pierce, I. J. Mercier, A. R. Lachmansingh, L. M. Pomeroy, E. A. Weck, D. Mascarenhas, in *UCLA Symposia on Molecular and Cellular Biology, New Series* (Liss, New York, 1987), vol. 62, pp. 301–310.
10. C. Rhodes, K. Lowe, K. Ruby, *BioTechnology* 6, 56 (1988).
11. T. Murashige and F. Skoog, *Physiol. Plant.* 15, 473 (1962).
12. H. Guilley *et al.*, *Cell* 30, 763 (1982).
13. R. F. Barker, K. B. Idler, D. V. Thompson, J. D. Kemp, *Plant Mol. Biol.* 2, 335 (1983).

14. M. Fromm, L. P. Taylor, V. Walbot, *Proc. Natl. Acad. Sci. U.S.A.* 82, 5824 (1985).
15. B. Reiss, R. Sprengel, H. Will, H. Schaller, *Gene* 30, 211 (1984).
16. I. J. Mettler, A. R. Lachmansingh, J. J. Detmer, D. A. Pierce, D. Mascarenhas, unpublished data.
17. C. A. Rhodes, R. L. Phillips, C. E. Green, *Can. J. Genet. Cytol.* 28, 374 (1986).
18. S. Edallo, C. Zucchinali, M. Perenzin, F. Salamini, *Maydica* 26, 39 (1981).
19. W. Werr, W. B. Frommer, C. Maas, P. Starlinger, *EMBO J.* 4, 1373 (1985).
20. S. Dellaporta, J. Wood, J. Hicks, *Plant Mol. Biol. Rep.* 1 (1983).
21. G. Schroder and J. Schroder, *Mol. Gen. Genet.* 185, 51 (1982).
22. E. Southern, *J. Mol. Biol.* 98, 503 (1975).
23. We thank our co-workers at Stauffer Chemical Co., especially R. Lachmansingh, K. Lowe, P. Olson, and L. Pomeroy, for their many contributions to this research. We also appreciate the assistance of D. Gray with the illustrations.

8 December 1987; accepted 29 February 1988

# Insecticidal Activity and Lectin Homology of Arcelin Seed Protein

THOMAS C. OSBORN, DANNY C. ALEXANDER,* SAMUEL S. M. SUN,† CESAR CARDONA, FREDRICK A. BLISS

**Arcelin, a major seed protein discovered in wild beans (*Phaseolus vulgaris*), has toxic effects on an important bean bruchid pest, *Zabrotes subfasciatus*. Transfer of the arcelin-1 allele to bean cultivars and addition of purified arcelin to artificial seeds results in high levels of insect resistance. The nucleotide and derived amino acid sequences of the arcelin-1 complementary DNA are very similar to those of genes encoding the bean seed lectin, phytohemagglutinin. The gene or genes encoding arcelin may have evolved from a phytohemagglutinin gene or genes resulting in an effective mechanism for resistance to bean bruchids.**

PLANTS HAVE EVOLVED WITH VARIous mechanisms to protect their seeds from insect predators. Proteins, which are major components of legume seeds, represent potential antibiosis factors that could affect predation (*1–3*). Seeds of common bean, *Phaseolus vulgaris* L., contain a carbohydrate-binding lectin protein called phytohemagglutinin (PHA). Although the function of PHA has not been demonstrated conclusively (*4*), Janzen *et al.* (*2*) suggested that a major part of its adaptive significance is to protect bean seeds from insect predators. That conclusion was based on the toxic effects of PHA on the cowpea weevil (*Callosobruchus maculatus* F.) when PHA was incorporated into artificial cowpea seeds.

Although PHA may protect bean seeds from predation by some insects, it is ineffective against the two most important bruchid pests of bean, the bean weevil, *Acanthoscelides obtectus* (Say), and the Mexican bean weevil, *Zabrotes subfasciatus* (Boheman). Most bean cultivars contain PHA, but no high levels of resistance have been found among cultivated materials (*5*). Among wild beans, however, accessions with high levels of resistance to these bruchid species have been identified (*6*). These wild accessions also contain a major seed protein, named arcelin, which has not been detected in seeds of bean cultivars (*7*). Four arcelin variants have been identified in wild beans. Accessions containing arcelin-2, -3, or -4 are resistant to the two bruchid species, but arcelin-1–containing accessions have not been identified as resistant, probably because the arcelin-1 allele occurs at low frequencies in wild accessions containing this variant (*6, 7*). In earlier studies, we found that genes controlling arcelin and PHA expression are tightly linked (*7*) and that arcelin-1 has several properties in common with PHA (*8*). In this study we report on insecticidal activity of the arcelin-1 protein in backcross-derived bean lines and in artificial seeds. We also report on the cloning and sequencing of a complementary DNA (cDNA) for arcelin-1 and on comparisons of arcelin and lectin sequences.

Although the presence of arcelin is correlated with bruchid resistance in wild beans, factors other than arcelin protein might confer the resistance property. To test whether resistance is associated with the genetic transfer of arcelin, we introduced the arcelin-1 allele from the wild line UW325 (*9*) into the bean cultivar Sanilac by two generations of backcrossing followed by two selfing generations. The expression of arcelin is controlled by a single Mendelian gene, and the presence of arcelin is dominant to its absence (*7, 9*). Seeds of backcross lines were tested for resistance to *Z. subfasciatus* (Table 1). On the basis of days until adult emergence and percentage emergence of adults, all arcelin-1–containing lines showed high levels of resistance. Lines lacking arcelin-1 were fully susceptible compared to the check cultivar, and lines segregating for arcelin-1 had intermediate levels of resistance. These results demonstrate that the arcelin-1 variant is associated with high levels of resistance to *Z. subfasciatus*. They also indicate that resistance is associated with the genetic transfer of arcelin-1 expression.

Analogous sets of backcross lines were developed from different cultivated bean types (for example, Pinto and black-seeded types) as recurrent parents. When these lines

**Table 1.** Levels of resistance to *Z. subfasciatus* in 'Sanilac' backcross-derived lines with arcelin-1 ($Arc^1/Arc^1$), without arcelin-1 (arc/arc), and segregating for arcelin-1 ($Arc^1/arc$). Lines were screened for resistance as described previously (*5*). Values represent the mean (±SEM) of two replicates containing 50 seeds each, each replication infested with seven insect pairs.

| Line or cultivar | Arcelin genotype | Days until adult emergence | Percentage emergence |
|---|---|---|---|
| Backcross line | | | |
| 3 | $Arc^1/Arc^1$ | 53.0 (±0.7) | 2.5 (±0.3) |
| 5 | $Arc^1/Arc^1$ | 47.8 (±3.2) | 2.1 (±0.2) |
| 4 | $Arc^1/arc$ | 33.2 (±2.3) | 20.9 (±5.6) |
| 7 | $Arc^1/arc$ | 37.2* | 38.7* |
| 8 | $Arc^1/arc$ | 38.1* | 34.6* |
| 9 | $Arc^1/arc$ | 35.4* | 30.2* |
| 1 | arc/arc | 34.2 (±0.2) | 89.5 (±3.8) |
| 2 | arc/arc | 34.7 (±0.1) | 76.3 (±1.5) |
| 6 | arc/arc | 34.4 (±0.3) | 93.8 (±6.2) |
| Susceptible cultivar | | | |
| Calima | arc/arc | 34.0 (±0.3) | 92.9 (±3.9) |

*Values based on one observation.

T. C. Osborn, Department of Agronomy, University of Wisconsin, Madison, WI 53706; formerly at ARCO Plant Cell Research Institute, 6560 Trinity Court, Dublin, CA 94568.
D. C. Alexander and S. S. M. Sun, ARCO Plant Cell Research Institute, 6560 Trinity Court, Dublin, CA 94568.
C. Cardona, Centro Internacional de Agricultura Tropical, AA 6713, Cali, Colombia.
F. A. Bliss, Department of Horticulture, University of Wisconsin, Madison, WI 53706.

*Present address: Calgene, 1920 5th Street, Davis, CA 95616.
†Present address: Department of Plant Molecular Physiology, University of Hawaii, Honolulu, HI 96822.